Vol. 85, No. 2, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 29, 1978 Pages 820-826

ASSOCIATION OF NUCLEOSIDEDIPHOSPHATE KINASE WITH MICROTUBULES[1]

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Received October 20, 1978

SUMMARY: A nucleosidediphosphate kinase activity (EC 2.7.4.6) which phosphorylates GDP to GTP is present in bovine brain microtubule protein prepared by cycles of assembly-disassembly. This activity persists through 5 cycles of assembly-disassembly and sediments with microtubules in sucrose density gradients, but is not associated with the tubulin dimer. It is proposed that the kinase is an integral part of the microtubule and is therefore a microtubule associated protein (MAP). Several isozymes of nucleosidediphosphate kinase exist in our preparations with a pI 7.6 form predominant. It may be speculated that this enzyme affects tubulin assembly in vivo by modulating the GTP/GDP ratio in the microtubule environment.

Tubulin prepared by the polymerization-depolymerization method of Shelanski et al. (1) has been shown to contain several enzymatic activities which may affect microtubule structure and function. Among these are a cAMP stimulated protein kinase activity which is capable of phosphorylating tubulin (2,3,4), a diglyceride kinase activity (5), a phospholipase C activity (6), and a tubulin α-subunit tyrosylating activity (7).

Of greater interest has been the interactions of tubulin and microtubule associated proteins (MAP) with non-cyclic nucleotides. The tubulin dimer has two guanine nucleotide binding sites, one readily exchanging GTP with the medium (E site) and the other (N site) binding GTP or GDP too tightly for significant exchange to occur (8). A transphosphorylation, converting GDP at the N site to GTP, can occur using either ATP or GTP as the phosphate donor. ATP is a better donor for this reaction than GTP although ATP does not bind to the

[1]Supported by Grants HD 06007 and AM 10209, U.S. Public Health Service.

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0006-291X/78/0852-0820$01.00/0

E site of the dimer. This led Jacobs, Smith, and Taylor (8) to hypothesize the existence of transphosphorylating enzymes in tubulin preparations that might use the dimer-GDP complex as a substrate. Although GTP has usually been used to promote *in vitro* tubulin assembly, it has sometimes been observed that ATP can promote microtubule formation (9,10). Jacobs and Caplow (11) have shown that trace amounts of GDP are required for ATP induced tubulin polymerization and have attributed this to a transphosphorylation reaction in which GTP is formed. Yanagisawa, Hasegawa, and Mohri (12) have found nucleoside diphosphate kinase (ATP: nucleoside diphosphate phosphotransferase EC 2.7.4.6) activity in the flagella of sea urchin spermatazoa in which the microtubules have been preserved by glycerol treatment.

In this paper we show the association of NDPK with bovine brain microtubules, paritally characterize this enzyme, and speculate on its *in vivo* role in microtubule function.

MATERIALS AND METHODS

The sodium salts of NADH, dGDP, ATP, GTP, GDP, and phosphoenolpyruvic acid as well as pyruvate kinase (EC 2.7.1.40) were purchased from the Sigma Chemical Company. The sodium salt of dTDP, lactate dehydrogenase (EC 1.1.1.27) and NDPK were purchased from Boehringer Mannheim Biochemicals. [γ-^{32}P]ATP was purchased from the Amersham Corporation.

Tubulin preparation. Tubulin was routinely prepared by a modification of the method of Shelanski *et al.* (1). A steer brain (400-450 g) was cooled, trimmed of blood clots and meninges, and homogenized in 0.5 ml of cold assembly buffer (AB) per g of tissue. AB is 20 mM MES pH 6.4, 70 mM NaCl, 1 mM EGTA, and 0.5 mM $MgCl_2$. The homogenate was centrifuged at 4°C for 45 min at 25,000 g and for 45 min at 78,000 g. The supernatant was mixed with an equal volume of AB with 8 M glycerol. This mixture was then made 0.5 mM ATP and 0.5 mM GTP, incubated at 37°C for 45 min, and centrifuged at 78,000 g for 1 hr at 30°C. The resuspended pellets were used as cycle 1 tubulin. All further cycles were done similiarly except that all centrifugation steps were done at 105,000 g for 1 hr. Cycle 2 tubulin, which is used for most of the experiments, is judged to be greater than 90% pure tubulin by sodium dodecylsulfate-polyacrylamide gel electrophoresis and Commassie blue staining (13). Protein concentrations were determined by the method of Lowry *et al.* (14), as automated in this laboratory for use with a Gilford 3500 automated analyzer (15).

In vitro microtubule assembly. *In vitro* polymerization of tubulin was done in AB with cycle 2 tubulin at a final concentration of 2-6 mg/ml. Before use, cycle 2 tubulin was centrifuged at 6,500 g for 5 min at 4°C to remove particles in order to reduce background light scattering. Samples were made in the cold and placed in the 37°C water-jacketed chamber of a Gilford 2400 multisample spectrophotometer. Nucleotide additions were made just before assembly was begun. Assembly was followed by measuring light scattering at 350 nm (16).

Nucleosidediphosphate kinase assays. Nucleosidediphosphate kinase (NDPK) was assayed by a coupled enzyme system similiar to that of Bergmeyer (17). The assay mixture contained 83.3 mM triethanolamine pH 7.5, 16.7 mM $MgCl_2$, 67 mM KCl, 1.1 mM PEP, 4.4 mM ATP, 0.2 mM NADH, 0.2 units/ml pyruvate kinase, 9 units/ml lactate dehydrogenase, and 0.7 mM dTDP (or 0.32 mM dGDP). The reaction was followed by decreasing absorbancy at 340 nm in a Gilford 2400 multisample spectrophotometer at 30°C. The reaction was started without the deoxynucleosidediphosphate, so the initial rate obtained corresponded to ATPase present in tubulin preparations and was subtracted from the reaction rate after dTDP (or GDP) was added. There is also a background rate (without NDPK) correction in this assay since dTDP and dGDP are substrates, though poor ones, for pyruvate kinase.

Isotope assays for NDPK were done by a method similar to that of Nakai and Glinsman (18). For some assays the reaction was stopped by the addition of 10% TCA. Protein was pelleted at 4°C, and the supernatant was extracted 3 times with 3 volumes of diethyl ether and lyophilized. The lyophilized nucleotides were picked up in a small volume of distilled water and spotted in PEI cellulose plates.

Sucrose density gradient centrifugation. Gradients of 30-60% sucrose in 10 mM MES, pH 6.5, and 1 mM $MgCl_2$ were formed by allowing 11.5 ml discontinuous gradients of 60%, 50%, 40%, and 30% sucrose in equal volumes to diffuse at 35°C for 8 hr. The gradients were then cooled to 30°C. A pellet of twice polymerized Shelanski tubulin was resuspended in 50 mM MES pH 6.5, 1 mM $MgCl_2$, and 10% sucrose at 30°C and then centrifuged at 8,000 g for 10 min at room temperature to remove particles. Samples (0.5 ml) of the supernatant were layered on gradients and centrifuged at 38,000 rpm in a Beckman SW41 rotor for 1 hr at 30°C. Immediately after the run, 0.5 ml fractions were removed successively from the top of each gradient with a syringe. Each fraction was assayed for NDPK activity by the coupled enzyme assay and for protein concentration.

Isoelectric focusing. Isoelectric focusing of cycle 2 tubulin was performed by the method of Behnke et al. (19). Sucrose gradients (5 ml) containing cycle 2 tubulin and Ampholine ampholytes (pH 3-10) were supported in tubes on plugs of 7.5% polyacrylamide. They were focused at constant voltage (150 V) in an ISCO gel electrophoresis apparatus. Soon after the voltage was removed, 0.2 ml fractions were removed successively from the top of each gradient. Fractions were assayed for NDPK activity by the coupled enzyme method (dTDP), and the pH of each fraction was determined.

RESULTS AND DISCUSSION

The results of in vitro polymerization of cycle 2 tubulin are shown in figure 1. When nucleotide was added just as the polymerization was begun (A), there was a time lag between the onset of GTP induced and ATP induced assembly. This time lag may correspond to the time required for NDPK to catalyze the production of enough GTP from ATP and GDP for polymerization to proceed. When there is a pre-incubation with ATP or GTP for 15 min at 15°C, this time lag is eliminated (B). During this pre-incubation, enough GTP may have been formed by the action of NDPK to support immediate polymerization. With or without a

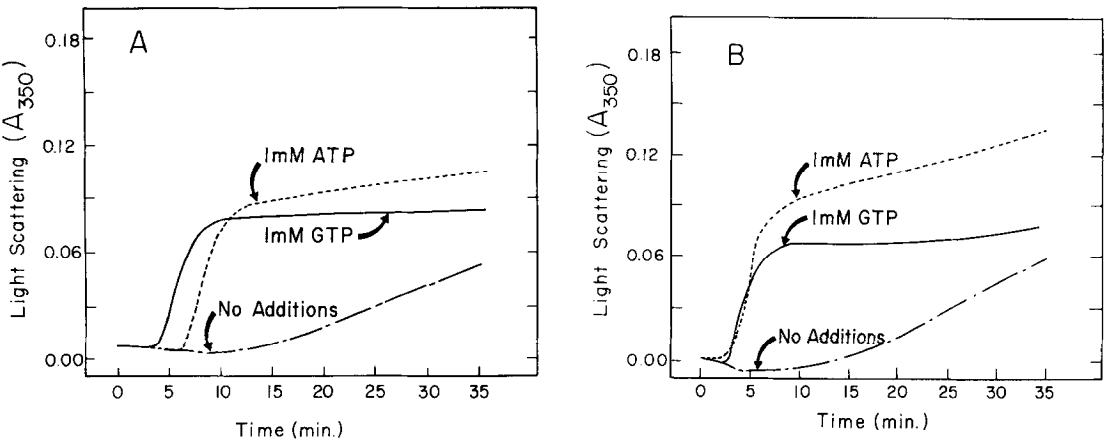
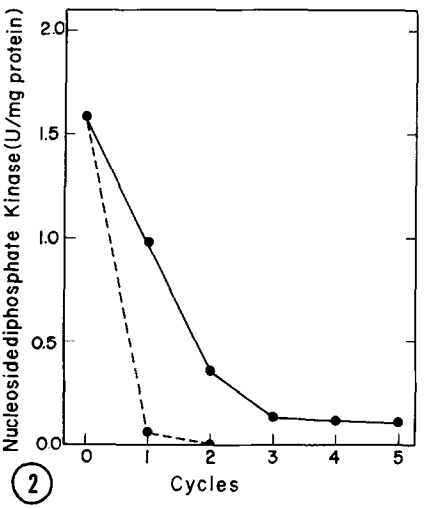


Figure 1. In vitro polymerization of bovine brain tubulin in assembly buffer was followed by measuring light scattering at 350 nm and 37°C in a Gilford 2400 spectrophotometer. In experiment A, nucleotide was added just as polymerization began. In experiment B, all polymerization mixtures were the same as in run A, but nucleotides were added and the samples were incubated at 15°C for 15 min before polymerization. All samples contained 5.6 mg/ml cycle 2 protein.

nucleotide pre-incubation, ATP produced more polymerization than GTP. This can be attributed to GDP inhibition of tubulin assembly. GDP can be eliminated through the NDPK reaction when ATP is the phosphate donor but not when GTP is the donor, due to a futile exchange of the γ-phosphate. Because the choice of nucleotide affects the time at which polymerization begins but not the rate at which it proceeds, it may be a nucleation event and not assembly that is affected by NDPK activity.

NDPK assays by both the coupled enzyme and isotope methods confirmed the presence of NDPK activity in our tubulin preparations. This activity was not associated with the dimer itself. In AB, tubulin eluted from a phosphocellulose column in the void volume; NDPK did not.

Steer brain tubulin was purified through 5 cycles of assembly-disassembly. The material from each cycle was assayed for NDPK by the coupled-enzyme assay (dGDP) and for protein. The results, presented in figure 2, show NDPK activity decreasing rapidly through the first few cycles but more gradually than would be predicted by simple dilution. By cycle 3, the specific activity stabilizes



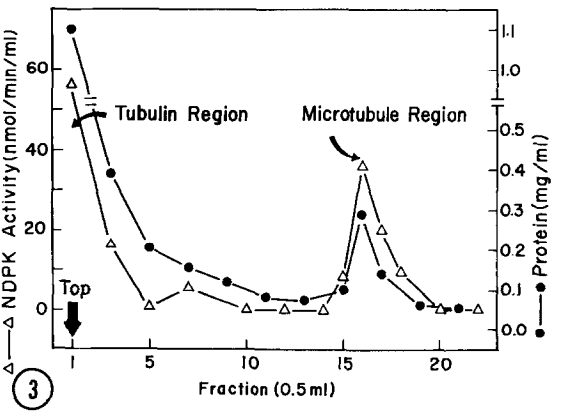


Figure 2. Bovine brain tubulin was purified through 5 assembly-disassembly cycles. The upper curve (solid line) shows the nucleosidediphosphate kinase specific activity after each cycle. The lower curve (broken line) shows the highest estimate of enzyme specific activity expected by simple dilution in each cycle.

Figure 3. Sucrose gradient centrifugation of twice polymerized bovine brain tubulin. Samples were centrifuged in 30-60% sucrose in 10 mM buffer, pH 6.5, and 1 mM $MgCl_2$ at 38,000 rpm in a Beckman SW41 rotor for 1 hr at 30°C. Each fraction was assayed for nucleosidediphosphate kinase and protein as described in the Material and Methods section.

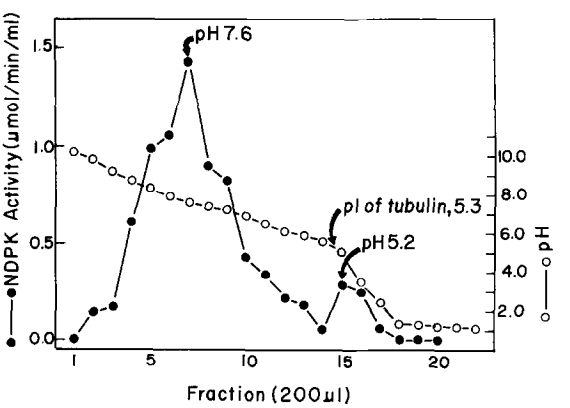


Figure 4. Isoelectric focusing of twice polymerized tubulin at constant voltage (150 V) for 18 hr. Fractions were assayed for nucleosidediphosphate kinase and pH.

at a value that is constant through subsequent cycles. In this manner, NDPK behaves like, and may be identified as, a microtubule associated protein.

Sucrose density gradient centrifugation of intact microtubules (figure 3) resulted in some NDPK activity co-sedimenting with a band of microtubules. The specific activity of NDPK in this microtubule region was greater than in the top few fractions where commercially obtained NDPK sedimented on an identical gradient in the absence of tubulin. NDPK may be an integral constituent of the microtubule.

The heterogeneity of NDPK in many tissues has been reported (20,21). It was therefore important to establish which isozymes were present in our tubulin preparations. The results of an isoelectric focusing experiment are shown in figure 4. The predominant form of NDPK in cycle 2 tubulin preparations had a pI of 7.6. A second peak of activity was observed in the pH region where tubulin focuses (pH 5.2). It is possible that opposite charge plays a role in the interaction of polymerized tubulin with the predominant form of NDPK.

REFERENCES

1. Shelanski, M.L., Gaskin, F., and Cantor, C.R. (1973) Proc. Nat. Acad. Sci. 70, 765-768.
2. Goodman, D.B.P., Rasmussen, H., DiBella, F., and Guthrow, C.E. (1970) Proc. Nat. Acad. Sci. 67, 652-659.
3. Lagnado, J.R., Lyons, C.A., Weller, M., and Phillipson, O. (1972) Biochem. J. 128, 95p.
4. Shigekawa, B.L., and Olsen, R.W. (1975) Biochem. Biophys. Res. Commun. 63, 455-462.
5. Daleo, G.R., Piras, M.M., and Piras, R. (1974) Biochem. Biophys. Res. Commun. 61, 1043-1050.
6. Quinn, P.J. (1973) Biochem. J. 133, 273-281.
7. Raybin, D., and Flavin, M. (1977) Biochem. Biophys. Res. Commun. 65, 1088-1095.
8. Jacobs, M., Smith, H., and Taylor, E.W. (1974) J. Mol. Biol. 89, 455-468.
9. Weisenberg, R.C. (1972) Science 177, 1104-1105.
10. Gaskin, F., Cantor, C.R., and Shelanski, M.L. (1974) J. Mol. Biol. 89, 737-758.
11. Jacobs, M., and Caplow, M. (1976) Biochem. Biophys. Res. Commun. 68, 127-135.
12. Yanagisawa, T., Hasegawa, S., and Mohri, H. (1968) Exp. Cell Res. 52, 86-100.
13. Weber, K., and Osborn, M. (1969) J. Biol. Chem. 244, 4406-4412.
14. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
15. Mak, I.T., and Wells, W.W. (1977) Arch. Biochem. Biophys. 183, 38-47.

16. Berne, B.J. (1974) J. Mol. Biol. 89, 755-758.
17. Bergmeyer, H.U. (1974) Methods of Enzymatic Analysis. New York: Academic Press, pp. 488-489.
18. Nakai, C., and Glinsmann, W. (1977) Biochem. Biophys. Res. Commun. 74, 1419-1425.
19. Behnke, J.N., Dagher, S.M., Massey, T.H., and Deal, W.C. (1975) Anal. Biochem. 69, 1-9.
20. Cheng, Y.C., Agarwal, R.P., and Parks, R.E. (1971) Biochemistry 10, 2139-2143.
21. Cheng, Y.C., Robison, B., and Parks, R.E. (1973) Biochemistry 12, 5-10.